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(54) Title: NEW PHARMACEUTICAL PREPARATIONS, CONTAINING 8-CHLORO-3(betaDIETHYLAMINOETHYL)-4-METHYL-7-ETHOXYCARBONYLMETHOXY COUMARIN AND THE SALTS THEREOF, IN THE TREATMENT OF PATHOLOGICAL CONDITIONS INVOLVING HIGH RELEASE OF NITRIC OXIDE

(57) Abstract

A pharmaceutical composition which comprises as an active ingredient, an effective amount of 8-chloro-3(beta-diethylaminoethyl)-4-methyl-7-ethoxycarbonylmethoxy coumarin or a pharmaceutically acceptable salt thereof, for the treatment of pathological conditions involving high release of nitric oxide (NO).

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"NEW PHARMACEUTICAL PREPARATIONS, CONTAINING 8-CHLORO-3 (betaDIETHYLAMINOETHYL)-4-METHYL-7 ETHOXYCARBONYLMETHOXY COUMARIN AND THE SALTS THEREOF, IN THE TREATMENT OF PATHOLOGICAL CONDITIONS INVOLVING HIGH RELEASE OF NITRIC OXIDE"

FIELD OF THE INVENTION

The present invention concerns pharmaceutical preparations, containing cloricromene (8-chloro-3(beta-diethylaminoethyl)-4-methyl-7-ethoxycarbonylmethoxy coumarin) and the salts thereof, in the treatment of diseases involving high release of nitric oxide (NO); specifically, the pathological conditions wherein this compound exerts an anti-inflammatory and/or immunosuppressive activity, or, more in general, the pathological conditions involving vasodilation and/or tissue damage derived from NO overproduction.

DESCRIPTION OF RELATED ART

1. Cloricromene.

It is well known that pure cloricromene was obtained for the first time using highly sophisticated methods, as described by the Applicant (US 4,296,039, US 4,452,811). In particular, it has been shown that the selective halogenation of a chlorine atom in position 8 of the coumarin molecule has the advantage of conferring remarkable vasodilator, in addition to antiarrhythmic (US 4,349,566) and anti-platelet aggregation (US 4,302,741) properties to the said compound.

The effects of cloricromene, both in vitro and in vivo, have been widely demonstrated in different experimental models. In particular, it has been shown that the compound exhibits a series of interesting activities on platelets, which turn into prevention of platelet activation and aggregation depending on different stimuli, such as arachidonic acid, collagen, ADP, adrenaline or platelet-activating factor (PAF), or on a combination of stimuli (Galli et al.: "Effects of 8-monochloro-3-beta-diethylaminoethyl-4-methyl-7ethoxy

carbonyl methoxy coumarin and thromboxane B2 formation in human platelets". Pharmacol. Res. Commun. 1980, 12:329-33; Prosdocimi M. et al.: "Action of AD6 (8-monochloro-3-betadiethylaminoethyl-4-methyl-7ethoxy carbonylmethoxy coumarin) on human platelets in vivo". Naunyn Schmiederberg's Arch. Pharmacol. 1986, 332:305-310; Travagli R.A. et al.: "Molecular aspects of cloricromene (AD6) distribution in human platelets and its pharmacological effect". Thromb. Res. 1989, 54:327-338). In addition, evidence has been shown that the remarkable inhibitory activity of AD6 at the level of production of the arachidonic acid, a precursor of the thromboxane synthesis, occurs likely through a specific inhibition of phospholipase A_2 activity (Porcellati S. et al. "The coumarin derivative AD6 inhibits the release of arachidonic acid by interfering with phospholipase A_2 activity in human platelets stimulated with thrombin". Agents & Actions 1990, 29:364-373).

Recently, it has been demonstrated that cloricromene is also able to inhibit polymorphonuclear cell adhesion to endothelial cells (Bertocchi et al. "In vitro inhibition of human polymorphonuclear cell function by cloricromene". Naunyn-Schmiedeberg's Arch. Pharmacol. 1989, 329:697-703). Moreover, the compound may have beneficial effects at the level of biochemical interactions between platelets and polymorphonuclear leukocytes (Zatta A. et al.: "Polymorphonuclear leukocyte-dependent modulation of platelet function: effect of cloricromene". Eur. J. Pharmacol. 1991, 198:97-100), which are known to be relevant in thrombotic and ischemic conditions.

In parallel, the efficacy of cloricromene in different experimental models in vivo has been investigated. In particular, it has been demonstrated that the compound exerts anti-thrombotic activity where a critical arterial stenosis is induced (Prosdocimi M.

et al. "Stenosis and vascular damage as an experimental model of arterial thrombosis: a role for prostanoids". In: Samuelsson et al. eds., Prostanoids and drugs. Plenum Publishing Corporation, 1989: 113-119; Prosdocimi M. et al. "Inhibition by AD6 (monochloro3-beta-diethylaminoethyl-4-methyl-7-ethoxycarbonylmethoxy coumarin) of platelet aggregation in dog stenosed coronary artery". Thromb. Res. 1985: 39:399-40).

Thus, from the evaluation of the current knowledge on cloricromene, the therapeutic use of this compound in all situations involving vasodilation and tissue damage induced by nitric oxide (NO) has never been proposed, such as for example, pulmonary inflammation, oedema, erythema, dermatitis, psoriasis, skin ulcers, arthrosis, rheumatoid arthritis and other autoimmune diseases, hypotensive shock, septic shock, hypovolemic shock; vascular diseases, such as inflammations derived from thrombophlebitis, hemorrhoids, ulcerative colitis, or, more in general, in those pathological situations characterized by vasodilation and/or tissue damage involving an overproduction of nitric oxide.

2. Biological role of nitric oxide (NO).

The identification of nitric oxide in mammalian tissues, and the comprehension of its biological role have been thoroughly investigated over the last decade. Nitric oxide has a potent vasodilator activity, it is synthesized in blood vessels by the action of two different NO synthases which utilize L-arginine as a substrate. One of these two enzymes is always present in the endothelium of blood vessels, both in animals and humans, in two isoforms, wherein it synthesizes low NO concentrations which, in turn, activate guanylate cyclase in the vascular smooth muscles. Such an enzyme is responsible for the maintenance of the vascular tone and it provides the physiological control of blood pressure (Vallance P. et al. "Effects of

endothelium-derived nitric oxide on peripheral arteriolar tone in man" Lancet 1989, 97-100). The second NO synthase occurs in the animal vascular endothelium, in the human vascular smooth muscle cells, in macrophages, and in other human cells by means of bacterial endotoxins and some cytokines. In vitro studies have demonstrated that the activation of such an enzyme yields a prolonged and massive NO synthesis, which in turn brings forth to a constant vasodilation and low reactivity to vasoconstrictive agents, associated with NO-induced damage. In such conditions, therapies containing L-arginine analogues, such as N^G-monomethyl-L-arginine (L-NMMA), which inhibits both types of NO synthases and may be selective for the NO-induced tissue damage, have proven effective (Gross S.S. et al. "Macrophage and endothelial nitric oxide synthesis: cell type selective inhibition by N^G-aminoarginine, N^G-nitroarginine, and N^G-methylarginine". Biochem. Biophys. Res. Commun. 1990, 170:96-103). Interesting results have been obtained also using glucocorticoids (Rees R.D. et al. "Dexamethazone prevents the induction by endotoxin of a nitric oxide synthase and the associated effects on vascular tone: an insight into endotoxin shock" Biochem. Biophys. Res. Commun. 1990, 173:541-547).

It is also to be considered that the NO synthase enzyme has been identified even in other systems, such as for example the nervous system - both central and peripheral nervous system, sensory and motor (Schmidt H.H. et al "Enzymatic formation of nitrogen oxides from L-arginine in bovine brain cytosol" Biochem. Biophys. Res. Comm. 1989, 165:284-291; Murphy S. et al. "Evidence for an astrocyte-derived vasorelaxing factor with properties similar to nitric oxide" J. Neurochem. 1990, 55:349-351), in the visual system, at the level of retina (Ross C.A. et al. "Messenger molecules in the cerebellum". Trends. Neurosci. 11990, 3:216-222),

wherein it could explain the pathogenetic mechanisms underlying some diseases affecting this anatomical region (Lolley R.N. et al. "Cyclic GMP accumulation causes degeneration of photoreceptor cells: stimulation of an inherited disease" Science 1977, 196:664-666). Therefore, the observed activation of such an enzyme, not only in cells belonging to the endothelial reticulate system, but also in several other cells and tissues, constitutes outward evidence that the NO release may play a major biological role, thus determining pathological conditions of vasodilation and tissue damage. In addition, also unspecified immunity situations could be associated with the NO synthase activation. Immunologically derived NO, besides being cytostatic or cytotoxic for pathogenic microorganisms and tumoral cells, could have negative effects on host cells forced to express NO synthase, or in adjacent cells. In fact, macrophages, hepatocytes and adenocarcinoma cells wherein NO was induced, have shown signs of related toxicity (Albina J.E. et al. "Regulation of macrophage physiology by L-arginine: role of the oxidative Larginine deiminase pathway" J. Immunol. 1989, 143:3641-3646; Billiar T.ER. et al. "An L-arginine dependent mechanism mediates Kuppfer cell inhibition of hepatocyte protein synthesis in vitro". J. Eng. Med. 1989, 189:1467-1472; O'Connor K.J. et al." Glucocorticoids inhibit the induction of nitric oxide synthase and the related cell damage in adenocarcinoma cells". Biochim. Biophys. Acta, submitted 1991).

The biological effects of such modifications, as well as the situations wherein NO release brings forth dysfunctions and/or cell death, have to be further investigated. Nevertheless, conditions of local or systemic tissue damage, associated with immunological situations, could have occurred in close parallel with NO release. Nitric oxide, besides the effects on cell viability and proliferation, may also play a major role

in the normal regulation of cell response to mitogens. At the moment, we do not know whether NO, derived from inducible enzyme, may contribute in the cytotoxic activity of other cells which play a role in specific immunity, though the activation of NO synthase in T-lymphocytes has been demonstrated (Kir K.S.J. et al "Cloned murine T-lymphocytes synthesize a molecule with the biological characteristics of nitric oxide" Biochem. Biophys. Res. Commun. 1990, 173:600-665).

From the above evidence, it results that nitric oxide - particularly the activation of NO synthase - plays a major role in various cell types, hence the crucial role of NO associated with pathological modifications affecting different tissues.

Among the pharmacological agents inhibiting NO synthase activation, special attention should be drawn on glucocorticoids. The discovery that these agents inhibit the activation of such an enzyme substantiates the potential role of NO in various situations, such as for example pulmonary inflammation, oedema, erythema, dermatitis, psoriasis, skin ulcers; arthrosis, rheumatoid arthritis and other autoimmune diseases; septic shock, hypovolemic shock; vascular diseases, such as inflammations deriving from thrombophlebitis, hemorrhoids; ulcerative colitis or, more in general, in those pathological situations of vasodilation and/or tissue damage wherein an overproduction of nitric oxide is observed.

The above evidence substantiate, though for pure exemplary purposes, the remarkable therapeutic potential of pharmaceutical preparations which are effective in decreasing NO overproduction, and in particular in inhibiting the expression of inducible-type NO synthase.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the time-dependent loss of tone in phenylephrine-contracted aortic rings from naive (O) or

L PS shocked rats treated with cloricromene (●) or vehicle ( ). Cloricromene (2 mg/kg) or vehicle (1 ml/kg of 0.9% NaCl solution) were given intravenously 30 min. before LPS administration. Each point represents the mean ± S.E.M. of 6 experiments. * P < 0.01 vs. LPS-shocked rats treated with vehicle.

FIG. 2 shows the cumulative concentration-effect curves for phenylephrine in aortic rings from naive (o) or LPS shocked rats treated with cloricromene (●) or vehicle ( ). Cloricromene (2 mg/kg) or vehicle (1 ml/kg of 0.9% NaCl solution) were given intravenously 30 min. before LPS administration. Each point represents the mean ± S.E.M. of 6 experiments. * P < 0.05; ** P < 0.01 vs. LPS-shocked rats treated with vehicle.

FIG. 3 shows the effect of cloricromene on NO_2-generation by J774 cells stimulated with LPS (100 mg/ml). Each point represents the mean ± S.E.M. of 6-8 experiments.

FIG. 4 shows % inhibition of exudative dermatitis induced by Croton oil in the rat ear. Anti-edemic activity of AD6 (I.P. administration 10 minutes before the test).

FIG. 5 shows % inhibition of exudative dermatitis induced by Croton oil in the rat ear. Antiedemic activity of AD6 (local administration together with the Croton oil).

FIG. 5A shows granuloma pouch induced by Croton oil in the rat.

FIG. 6 shows % inhibition of peritonitis induced by acetic acid in the rat. Anti-exudative effect of AD6 (oral administration 1 hour before the test).

FIG. 7 shows % inhibition of peritonitis induced by acetic acid in the rat. Anti-exudative effects of AD6 (i.v. administration 5 minutes before the test).

FIG. 8 shows the time-course of the anti-exudative effect of AD6 (oral administration - 0, 1 mg/kg)

FIG. 8A shows % inhibition of serotonin induced paw edema by AD6.

FIGS. 9-14 show % inhibition of phenylquinone induced writhing in the mouse by AD6 through various doses and routes of administration.

DETAILED DESCRIPTION OF THE INVENTION

We have surprisingly found - which constitutes the object of the present invention - that cloricromene (8-chloro-3-(beta-diethylaminoethyl)-4-methyl-7-ethoxy carbonylmethoxy coumarin hydrochloride) is a potent inhibitor of NO synthase and may be therefore used to advantage in pharmaceutical preparations aimed at treating pathological situations involving nitric oxide (NO) overproduction, such as for example, pulmonary inflammation, oedema, erythema, dermatitis, psoriasis, skin ulcers; arthrosis, rheumatoid arthritis and other autoimmune diseases; septic shock, hypovolemic shock; vascular diseases, such as inflammations deriving from thrombophlebitis, hemorrhoids; ulcerative colitis or, more in general, in those pathological situations of vasodilation and/or tissue damage wherein an overproduction of nitric oxide is observed.

The object of the present invention results from the description of the experiments which were performed with cloricromene in the following experimental models:

1. rat isolated aortic rings, wherein the loss of tone induced by lipopolysaccharides (LPS) was measured;
2. mouse macrophage cultures treated with LPS;
3. exudative dermatitis induced by croton oil in the mouse;
4. granuloma pouch induced by croton oil in the rat;
5. peritonitis induced by acetic acid in the rat;
6. paw oedema induced by serotonin in the rat;

7. writhing test induced by phenylquinone in the mouse.

MATERIALS AND METHODS

Isolated aorta

Wistar male rats (weighing 280-320 g) were anesthetized with ether and i.v. injected with 4 mg/kg lipopolysaccharide (LPS) of Salmonella Thyphi. Cloricromene was i.v. injected (2 mg/kg, "in bolus") 30 minutes before LPS administration. Controls received a solution of NaCl (0.9% in 1 ml/kg), whereas "naive" rats received neither LPS nor cloricromene.

Thoracic aorta was then removed, 3 hrs. after LPS administration, and kept in Krebs solution, pH 7.4, containing (mM): NaCl: 11.4; KCl: 4.7; $MgSO_4$: 1.2; $CaCl_2$: 1.3; KH_2PO_4: 1.2; $NaHCO_3$: 25.0 and glucose: 11.7. Each aorta, upon proper removal of the adjacent adipose and connective tissue, was cut into rings of approximately 2 mm length. Rings were then kept in 10 ml oxygenated (95% O_2 and 5% CO_2) baths containing Krebs solution, at 37°C. Rings were then connected to a recording apparatus by means of isometric transducers: the "tone" was properly balanced with approx. 1 g tension for 90-120 min. Isometric tension was continuously monitored by means of an isometric transducer connected to a recording apparatus (Ugo Basile, Comerio, Varese, Italy). All experiments were performed in the presence of 10 μM indomethacine.

Subsequently, rings were contracted with phenylephrine (PE, 300 μM) and spontaneous vascular tone loss was evaluated for 4 hrs.

In a different set of experiments, aortic rings were contracted with increasing concentrations of PE (1 nM - 10 μM), to obtain cumulative dose/response curves.

Results (mean ± S.E.M.) were expressed as g of tension/mg of tissue.

Cell cultures

Rat macrophages (J774 cell line - American Tissue Culture catalogue T1B67) were cultured in Techne flasks, centrifuged at 25 rpm and incubated at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2% glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Cells were seeded on multiwell (24-well) plates (Falcon) at a density of $2.5x10^5$ cells and kept to adhesion in 5% CO_2-95% O_2 for 2 hrs. at 37°C. The culture medium was then substituted with fresh medium and cells were LPS-activated (100 nf/ml) and incubated with the tested compound. Cloricromene was tested at the following concentrations: 2, 20 and 200 μM.

NO production was then measured determining the amount of nitrites (NO_2-) in the culture medium, according to Griess (Di Rosa et al. "Glucocorticoids inhibit the induction of nitric oxide synthase in macrophages" Biochem. Biophys. Res. Commun. 1990, 172:1246).

Results are expressed as moles of NO_2 released from 10^6 cells, over a 24 hr. period. The statistical analysis, for both tests, was performed using the Student-t test.

Anti-inflammatory activity of AD6 (systemic administration, both i.p. and local) in exudative dermatitis induced by croton oil in the mouse.

The test consisted in the induction of an exudative dermatitis in the mouse auricle, by instillation of a skin irritating agent - croton oil - so as to cause a specific "vascular" inflammatory reaction, characterized by hyperemia and oedema. The inflammatory reaction, characterized by slow evolution and long duration, affects the deep dermal layers, wherein it extends gradually from the application site to the whole auricle, reaching its maximum intensity at the 6th hour. The pathogenic mechanisms responsible for the vascular

and exudative phenomena acknowledge the role of several factors (activation of protein kynase C, of the kininogen system and the complement, release of lysosomial enzymes, vasoactive autacoids, prostaglandins and chemotaxic factors; cell infiltration, collagen modification). Mice weighing 28-30 g were divided into homogeneous groups of 6 animals. The method according to Tubaro et al. was used (Agents & Actions 1985, 17:197). Dermatitis was induced in the anesthetized mouse (pentobarbital, 37 mg/kg, i.p.) by instillation on the inner surface of the right ear of a solution containing 35 μg croton oil in 15 μl acetone. After 6 hrs., mice were sacrificed with ether in order to cut the edematous auricle (dx) and the healthy contralateral one (sx). The effect of AD6 was studied, both by systemic administration (i.p., in 10 ml/kg saline) and local application (vehiculating the substance directly in the croton oil/acetone solution). The effects of the treatment were evaluated on the basis of a weight increase of the treated ear with respect to the contralateral one, 6 hrs. after instillation of the irritant, thus assuming the percentage inhibition with respect to controls as an index of the anti-inflammatory activity.

Mean comparisons were done by means of the Student-t test. DE_{40} was measured on the dose/response curve referring to the percentage inhibition of the oedema, according to Litchfield and Wilcoxon (J. Pharmac. Exp. Therap. 1949, 96:99).

Chronic anti-inflammatory activity of AD6 (i.p. administration) in the granuloma pouch induced by croton oil in the rat.

The experiments consisted in the induction of a subacute local inflammatory reaction in the rat back subcutaneous tissue, by means of air inoculation followed by a solution of croton oil in acetone. The experimental model allows to study two different aspects

of an inflammatory reaction which is very common in human pathologies, i.e. exudation and formation of granulomatous tissue, consisting of a granulomatous vesicle full of exudate.

The pathogenic mechanism is associated with the specific irritating action of croton oil (phorbol esters), which causes activation of protein kynase C, of the complement and the plasmic kininogen, which yields kinins (bradykinins), considerable exudation and leukocyte migration. The model of Seyle was used (J. Amer. Med. Ass., 1953, 152:1207), further modified by Finney & Somers (J. Pharm. Pharmac. 1958, 10:613), which consists in the subcutaneous administration of 25 ml air, followed by 0.5 ml of a solution of croton oil in 2% seed oil. Sprague-Dawley rats weighing 130-140 g were divided into homogeneous groups of 6 animals. AD6 and PBZ (phenylbutazone) were vehicled in 10 ml/kg saline. Intraperitoneal administration was done for 5 consecutive days as follows:

- control/saline 10 ml/kg/day
- AD6 0.05-0.1 mg/kg/day
- PBZ 50-100 mg/kg/day

At the end of treatment, 24 hrs. after the last administration, the animals were sacrificed with ether, and the exudate contained in the vesicle was removed, while the granulation tissue forming the vesicle wall was examined under a magnifying lens.

Phenylbutazone (PBZ), a non-steroid anti-inflammatory agent, particularly active in this experimental model, was used as test compound.

Anti-inflammatory activity of AD6 (oral - i.v. administration) in peritonitis induced by acetic acid in the rat.

We investigated the anti-inflammatory activity of AD6 using as an experimental model, the peritonitis induced by acetic acid in the rat, a model of acute inflammation of exudative type, basically associated

with local irritation, activation of proteases and release of kinins and prostaglandins.

The dose/effect and the time course of AD6 by oral and i.v. route were determined.

The model described by Arrigoni-Martelli was used (Boll. Chim. Farm. 1968, 107:29) which, on the basis of the measurement of the peritoneal exudate volume, allows to evaluate the protective activity of a drug against the development of an acute inflammatory reaction induced by i.p. injection of acetic acid (10 mg/kg of a 0.5% solution) in the rat.

Thirty minutes after injection of the acetic acid, the animals were sacrificed with ether and the peritoneal effusion collected by means of Pasteur pipettes was measured after laparotomy.

Male Sprague-Dawley rats weighing 200-300 g were divided into homogeneous groups of 5 animals.

AD6, in saline solution, was administered by oral (gavage) and i.v. route, as follows:

- 10 ml/kg (oral route)
- 1 ml/kg (i.v.)

Treatment was performed at T_{max} (defined within the limits of the tests concerning the kinetics of the analgesic effect), namely:

A)	<u>Oral administration</u>	(1 h before testing)
	- Controls	Saline 10 ml/kg
	- AD6	0.05-0.075 - 0.1-0.2 mg/kg
B)	<u>i.v. administration</u>	(5 min before testing)
	- Controls	Saline 1 ml/kg
	- AD6	0.025, 0.5, 0.075, 0.1, 0.2, 0.4 mg/kg
	- Proendotel	0.05 0.1 - 0.2 mg/kg

<u>Oedema of the paw induced by serotonin in the rat.</u>

Male Sprague-Dawley rats weighing 140-160 g were divided into homogeneous groups of 5 animals.

The experiment consisted in the induction of a local edematous reaction through the subplantal inoculation of serotonin. The reaction, characterized by rapid evolution, is associated with an increase in capillary permeability, basically depending on the direct vasomotor action of serotonin and the activation of plasmic kininogens (formation of bradykinins).

The oedema was induced in the right hindpaw by injecting 0.1 ml of a serotonin/creatinine sulfate 0.05% solution.

Paw oedema volume was measured by plethysmometry, before and 45 min after the irritant injection. The development of the oedema was evaluated on the basis of the increased volume of the paw with respect to baseline value.

The pharmacological treatment was performed i.p. (10 min before testing) and i.v. (5 min before testing); Phenylbutazone (PBZ) and cyproheptadine (CYP) were used as test compounds in the i.p. treatment.

A) <u>i.p. administration</u> (10 min before testing)

- Controls	Saline 10 ml/kg
- AD6	0.05, 0.075, 0.1, 0.15 mg/kg
- PBZ	100 mg/kg
- CYP	0.5 mg/kg

B) <u>i.v. administration</u> (5 min before testing)

- Controls	Saline 1 ml/kg
- AD6	0.01, 0.05, 0.1 mg/kg

<u>Analgesic activity of AD6 in the writhing test induced by phenylquinone in the mouse.</u>

Male Swiss mice weighing 28-30 g were divided into homogeneous groups of 6 animals/dosage.

The method described by Siegmund et al. was used (Proc. Soc. Exp. Biol. 1957, 95:729), which is based on the capability of a drug to antagonize the syndrome induced by i.p. injection of 0.25 ml of a 0.02% solution

of phenylquinone in 5% ethyl alcohol in the mouse. Pain symptomatology is characterized by intermittent contractions to the abdomen, with writhes starting approximately 3 min. after injection of the algogenic agent, and lasting approximately 120 min. Practically, the measurement of such symptomatology is done calculating the number of writhes exhibited by each animal in 5 min (from the 5th to the 10th min after injection of phenylquinone), that is, when the pain reaction is stronger and the abdominal writhes are closer and more constant.

AD6 was administered in saline solution, by oral (gavage), i.p. and i.v. route, as follows:

- 10 mg/kg (os and i.p.)
- 1 ml/kg (i.v.)

Treatment was performed at T_{max} (defined within the limits of the tests concerning the kinetics of the analgesic effect, cfr. Par. 2), that is:

A) Oral administration (1 h before testing)
- controls: saline 210 mg/kg
- AD6: 0.025, 0.2, 0.75, 0.1, 0.2 mg/kg

B) i.p. administration (5 min before testing)
- controls: saline 10 ml/kg
- AD6: 0.01, 0.25, 0.05, 0.75, 0.1, 0.2 mg/kg

C) i.v. administration (5 min before testing)
- controls: saline 1 ml/kg
- AD6: 0.0005, 0.001, 0.005, 0.01, 0.02, 0.1 mg/kg

PHARMACOKINETICS AND PHARMACODYNAMICS

The study was performed using the writhing test induced by phenylquinone in the mouse (cfr. Par. 1).

Male Swiss mice weighing 28-30 g were divided into homogeneous groups of 6 animals.

AD6 was administered by oral, i.p. and i.v. route at different intervals, before i.p. injection of the algogenic agent. At the different routes of administration, the dose corresponding to the highest effect was used, i.e.:

- 0.1 mg/kg (os and i.p.)
- 0.01 mg/kg (i.v.)

The time course of the analgesic effect of the compound was evaluated (see Tables 10-11-12 herein below).

RESULTS

Hereinafter are summarized the results of the experiments.

Effect on reactivity and vascular tone.

Treatment with LPS induces a time-dependent decrease of vascular tone in aortic rings precontracted with PE. This relaxation is significantly higher with respect to the aortic rings obtained from LPS-untreated animals.

The pharmacological treatment with cloricromene (2 mg/kg i.v., in bolus) significantly reduces the increased relaxation and decreases the sensitivity to PE in LPS-treated rats. In the animals receiving cloricromene, vasoconstriction induced by increasing concentrations of PE and maximal contractility are significantly higher (Fig. 1) and longer in time (Fig. 2), with respect to the control groups treated with LPS and saline (NaCl 0.0%).

Effect on NO_2- formation in macrophage cultures.

Treatment with LPS induces a significant increase in nitrite (NO_2-) production.

Co-treatment with cloricromene inhibits NO_2- production: the effect is concentration-dependent,

reaching an inhibition of about 48% in the presence of 200 μM of cloricromene (Fig. 3).

Effect on exudative dermatitis induced by croton oil in the mouse.

AD6 has a marked protective, dose-dependent activity against exudative dermatitis induced by instillation of croton oil in the mouse ear.

The effect is remarkable and statistically significant both after local application (together with the irritating agent) and i.p. administration (Tables 1-2, Figures 4-5).

Effect on the granuloma pouch induced by croton oil in the rat.

AD6, administered by i.p. route at doses of 0.05-0.1mg/kg/day for 5 consecutive days, exerts a significant anti-exudative/anti-granulomatous activity in this experimental model.

The effect obtained with a dose of 0.1 mg/kg overlaps that induced by 50 mg/kg of phenylbutazone (Table 3 - Fig. 5A).

Effect on peritonitis induced by acetic acid in the rat

When administered by oral and i.v. route, AD6 exerts an anti-inflammatory activity which is evident only at low dosage, within a range of 0.025 - 0.1 mg/kg.

For the different routes of administration, the highest effect (detected at T_{max}) is reached at the dose of 0.1 mg/kg, as follows:

- by oral route
- 33% (T_{max}: 1 h)
- by i.v. route
- 45% (T_{max}: 5 min)

(Tables 4-4A-4B) - Figures 6-7-8)

Table 1

EXUDATIVE DERMATITIS INDUCED BY CROTON OIL IN THE MOUSE

Treatment (i.p.)	Dose mg/kg	Weight increase *dx* vs *sx* ear	Inhibition %	p Student-t	DE_{40} mg/kg
Vehicle	- - -	28.83 ± 1.83	- - -	- - - - -	
AD6	0.01	23.43 ± 1.22	19	< 0.05	
	0.025	18.18 ± 1.81	37	< 0.01	0.03
	0.05	11.98 ± 1.28	58	< 0.001	(0.01-0.08)
	0.01	8.33 ± 0.47	71	< 0.001	
	0.02	19.48 ± 1.19	32	< 0.01	

Anti-oedematous activity of AD6 by i.p. administration.

Weight measurement of both oedematous (dx) and healthy (sx) ear 6 hrs after instillation of the croton oil.

N. 6 animals/group

Table 2

EXUDATIVE DERMATITIS INDUCED BY CROTON OIL IN THE MOUSE

"in situ" treatment	Dose mg/kg	Weight increase *dx* vs *sx* ear % ± S.E.	Inhibition %	p Student-t	DE_{40} mg/kg
Vehicle	- - -	28.57 ± 1.31	- - -	- - - - -	
AD6	0.05	21.08 ± 1.12	26	< 0.01	
	0.1	13.08 ± 1.14	54	< 0.001	0.07
	0.2	14.38 ± 0.64	49	< 0.001	

Anti-oedematous activity of local application of AD6 (+ croton oil).

Weight measurement of both oedematous (dx) and healthy (sx) ear 6 hrs after instillation of the croton oil.

Test compound: indomethacine

N. 6 animals/group

Table 3

GRANULOMA POUCH INDUCED BY CROTON OIL IN THE RAT

Treatment (i.p.)	Dose mg/kg	Exudate volume ml ± S.E.	Inhibition %	Macrosc. aspect
Vehicle	- - -	3.53 ± 0.31		considerable hyperemia thick wall
AD6	0.05 x 5 days	1.98 ± 0.08*	44	moderate hyperemia thin wall
	0.1 x 5 days	1.38 ± 0.06*	61	moderate hyperemia thin wall
PBZ	50 x 5 days	1.42 ± 0.08*	60	moderate hyperemia thin wall
	100 x 5 days	0.70 ± 0.06*	80	moderate hyperemia thin wall

* $p < 0.01$ Student-t test

Anti-oedematous/anti-granulomatous activity of AD6 by i.p. administration in the rat, for 5 consecutive days.
At the 6th day: sacrifice and exudate withdrawal collected in the subcutaneous sac - mascroscopic examination of the granulomatous wall.

Test compound: phenylbutazone (PBZ)

N. 6 animals/group

Table 4

PERITONITIS INDUCED BY ACETIC ACID IN THE RAT

Treatment (i.v.)	Dose mg/kg	Weight increase ml ± S.E.	Inhibition %	p Student-t
Vehicle	- - -	2.22 ± 0.07	- - -	- - - - -
AD6	0.025	2.00 ± 0.05	10	< 0.05
	0.05	1.90 ± 0.05	14	< 0.01
	0.075	1.62 ± 0.06	27	< 0.001
	0.1	1.22 ± 0.05	45	< 0.001
	0.2	1.68 ± 0.06	24	< 0.001
	0.4	2.06 ± 0.07	7	n.s.

Effect of i.v. administration of AD6, 5 min before i.p. injection of acetic acid (10 ml/kg, 0.5 % solution)

N. 5 animals/group

Table 4A

PERITONITIS INDUCED BY ACETIC ACID IN THE RAT

Treatment (oral route)	Dose mg/kg	Exudate volume ml ± S.E.	Inhibition %	p Student-t
Vehicle	- - -	2.22 ± 0.08	- - -	- - -
AD6	0.025	1.95 ± 0.08	12	< 0.05
	0.05	1.90 ± 0.07	14	< 0.05
	0.075	1.68 ± 0.09	24	< 0.01
	0.1	1.48 ± 0.06	33	< 0.001
	0.2	1.62 ± 0.06	27	< 0.001

Effect of oral administration of AD6, 1 hr before i.p. injection of acetic acid (10 ml/kg, 0.5 % solution)

N. 5 animals/group

Table 4B

PERITONITIS INDUCED BY ACETIC ACID IN THE RAT

Treatment (oral route)	Time (hrs)	Exudate volume ml ± S.E.	Inhibition %	p Student-t
Vehicle	0.16	2.30 ± 0.11	- -	
AD6		1.98 ± 0.07	14	< 0.05
Vehicle	0.5	2.16 ± 0.09	- -	
AD6		1.68 ± 0.07	22	< 0.01
Vehicle	1	2.22 ± 0.08	- -	
AD6		1.56 ± 0.06	33	< 0.001
Vehicle	2	2.24 ± 0.06	- -	
AD6		1.62 ± 0.06	27	< 0.001
Vehicle	3	2.30 ± 0.09	- -	
AD6		1.82 ± 0.07	21	< 0.01
Vehicle	5	2.28 ± 0.08	- -	
AD6		2.00 ± 0.08	12	n.s.
Vehicle	6	2.24 ± 0.07	- -	
AD6		2.12 ± 0.06	5	n.s.

Time course of the antiexudative activity of AD6 by oral administration in the rat, at different time intervals before testing.

Dosage: 0.1 mg/kg by oral route

N. 5 animals/group

Effect on paw oedema induced by serotonin in the rat

When administered by i.p. route, AD6 inhibits the onset of oedema induced by serotonin in a limited, but statistically significant manner, in a dose range between 0.05 and 0.1 mg/kg. The effect decreases by further increasing the dose. The inhibition reached at the highest active dose (0.1 mg/kg) was 39% with respect to controls.

Phenylbutazone (PBZ) (100 mg/kg) failed to exert any activity in this experimental model, whereas cyproheptadine (CYP) exerted its marked anti-serotonergic activity, determining a 78% inhibition of the oedema at a dose of 0.5 mg/kg.

By i.v. route, the anti-edematous activity of AD6 is lower with respect to i.p. administration.

The active dose range is between 0.01 and 0.05 mg/kg (highest response - 25%) (Tables 5-6 - Fig. 8A).

Analgesic activity of AD6

After oral and i.p. administration, AD6 exerts a moderate analgesic activity at the PQ writhing test, at doses between 0.01 and 0.1 mg/kg (Tables 7-8, Fig. 12-13).

The effect, highest at 0.1 mg/kg, decreases markedly by increasing the dosage (bell shape).

After i.v. administration, AD6 exerts a marked analgesic activity at doses 10 times lower than those active by oral and i.p. route (Table 9, Fig. 14). Using this route of administration, maximal activity, clearly higher than that by oral and i.p. route, is reached using a dose of 0.01 mg/kg.

Further dose increments reduce the effect, but in a more gradual and less marked way with respect to what observed by oral and i.p. route.

Table 5

OEDEMA OF THE PAW INDUCED BY SEROTONIN IN THE RAT

Treatment (i.p.)	Dose mg/kg	Exudate volume ml ± S.E.	Inhibition %	p Student-t
Vehicle	- - -	0.54 ± 0.03	- - -	- - -
AD6	0.05	0.48 ± 0.03	11	n.s.
	0.075	0.43 ± 0.02	20	< 0.05
	0.1	0.33 ± 0.02	39	< 0.01
	0.15	0.40 ± 0.02	26	< 0.01
PBZ	100	0.50 ± 0.03	7	n.s.
CYP	0.5	0.12 ± 0.02	78	< 0.01

Antioedematous activity of AD6 by i.p. administration in the rat, 10 min before testing.

Test compounds: Phenylbutazone (PBZ)
Cyproeptadine (CYP)

N. 5 animals/group (controls n = 10)

Table 6

EDEMA OF THE PAW INDUCED BY SEROTONIN IN THE RAT

Treatment (i.v.)	Dose mg/kg	Exudate volume ml ± S.E.	Inhibition %	p Student-t
Vehicle	- - -	3.48 ± 0.02	- - -	- - -
AD6	0.01	0.42 ± 0.03	13	< 0.05
	0.05	0.36 ± 0.02	25	< 0.01
	0.1	0.46 ± 0.02	4	n.s.

Effect of i.v. administration of AD6, 5 min before testing

N. 5 animals/group

Table 7

WRITHING TEST INDUCED BY PHENYLQUINONE IN THE MOUSE

Treatment (oral route)	Dose mg/kg	N° writhes ± S.E. in 5 min	Inhibition %	p Student-t	DE_{40} mg/kg
Vehicle	- - -	29.5 ± 1.02	- - -	- - -	
AD6	0.025	20.8 ± 0.90	29	< 0.001	
	0.05	19.0 ± 1.04	35	< 0.001	
	0.075	17.2 ± 0.75	42	< 0.001	0.07
	0.1	14.2 ± 0.78	51	< 0.001	
	0.2	18.8 ± 0.49	36	< 0.001	

Analgesic activity of AD6 by oral administration in the mouse, 1 hr before testing.

N. 6 animals/dose

Table 8

WRITHING TEST INDUCED BY PHENYLQUINONE IN THE MOUSE

Treatment (i.p.)	Dose mg/kg	N° writhes ± S.E. in 5 min	Inhibition %	p Student-t	DE_{40} mg/kg
Vehicle	- - -	30.2 ± 0.65	- - -	- - -	
AD6	0.01	23.2 ± 0.70	23	< 0.001	
	0.025	19.0 ± 1.04	37	< 0.001	
	0.05	16.6 ± 0.75	45	< 0.001	0.04
	0.075	15.0 ± 0.80	50	< 0.001	
	0.1	10.8 ± 0.70	64	< 0.001	
	0.2	13.8 ± 0.51	54	< 0.001	

Analgesic activity of AD6 by i.p. administration in the mouse, 5 min before testing.

N 6 animals/dose

Table 9

WRITHING TEST INDUCED BY PHENYLQUINONE IN THE MOUSE

Treatment (i.v.)	Dose mg/kg	N° writhes ± S.E. in 5 min	Inhibition %	p Student-t	DE_{50} (C.L.) mg/kg
Controls	- - -	29.3 ± 0.95	- - -	- - -	
AD6	0.0005	20.2 ± 0.87	30	< 0.001	
	0.001	16.5 ± 0.77	44	< 0.001	
	0.005	10.2 ± 0.70	65	< 0.001	0.0022
	0.01	6.3 ± 0.42	78	< 0.001	(0.0006-0.009)
	0.02	8.2 ± 0.60	72	< 0.001	
	0.1	11.7 ± 0.81	60	< 0.001	

Analgesic activity of AD6 by i.v. administration in the mouse, 5 min before testing.

N. 6 animals/dose

Maximum doses and activity using the different routes of administration are as follows:

- 0.1 mg/kg os
- 52% (T_{max}: 1 h)
- 0.1 mg/kg i.p.
- 64% (T_{max}: 5 min)
- 0.01 mg/kg i.v.
- 78% (T_{max}: 5 min)

Oral administration

The determination of the time course of AD6 analgesic effect (0.1 mg/kg) emphasizes an activity peak after 1 hr., which is maintained almost in plateau until the 3rd hr., followed by a relatively rapid decrease, with half reduction of the effect around the 5th hr. (Table 10, Fig. 9).

I.p. administration

Using this route of administration, AD6 effect (0.1 mg/kg) is almost immediately evident (T_{max} after 5 min) and tends to decrease with a biexponential course: quite rapid within the 1st hr. and slower in the following hrs. $T_{1/2}$ is approximately 2 hrs. (Table 11, Fig. 10).

I.v. administration

Using this route of administration, the activity of AD6 (0.01 mg/kg) is almost immediately evident (T_{max} after 5 min) and is maintained to values close to peak for about 30 min. Then, the effect decreases gradually, with a t1/2 of approximately 2 hrs. (Table 12, Fig. 11).

PHARMACEUTICAL PREPARATIONS

Cloricromene, (8-chloro-3-(betadiethylaminoethyl)-4-methyl-7-ethoxycarbonylmethoxy coumarin and the salts thereof used in the present invention can be administered both to humans and animals, alone or in

Table10

KINETICS OF THE ANALGESIC ACTIVITY IN THE MOUSE
(WRITHING TEST INDUCED BY PHENYLQUINONE)

Treatment (oral route)	Time (hrs)	Writhing (n° ± S.E.)	Inhibition %	p Student-t
Vehicle	0.5	30.0 ± 1.03	- -	
AD6		24.6 ± 0.80	18	< 0.01
Vehicle	1	29.5 ± 1.02	- -	
AD6		14.2 ± 0.78	52	< 0.001
Vehicle	2	29.8 ± 0.65	- -	
AD6		16.0 ± 0.85	46	< 0.001
Vehicle	3	30.0 ± 1.02	- -	
AD6		14.0 ± 0.63	53	< 0.001
Vehicle	4	29.8 ± 0.83	- -	
AD6		17.8 ± 0.82	40	< 0.01
Vehicle	6	30.5 ± 1.04	- -	
AD6		24.6 ± 1.19	19	< 0.01

Time course of the analgesic activity of AD6 by oral administration in the mouse at different intervals before testing

Dosage: 0.1 mg/kg, oral route

N. 6 animals/group

Table 11

KINETICS OF THE ANALGESIC ACTIVITY IN THE MOUSE
(WRITHING TEST INDUCED BY PHENYLQUINONE)

Treatment (i.p.)	Time (hrs)	Writhing (n° ± S.E.)	Inhibition %	p Student-t
Vehicle	0.08	30.2 ± 0.75	- -	
AD6		10.8 ± 0.70	64	< 0.001
Vehicle	0.16	29.8 ± 0.48	- -	
AD6		11.7 ± 0.67	61	< 0.001
Controls	0.5	29.5 ± 1.02	- -	
AD6		12.2 ± 1.08	59	< 0.001
Vehicle	1	29.7 ± 0.61	- -	
AD6		17.3 ± 0.41	41	< 0.001
Vehicle	2	29.8 ± 0.95	- -	
AD6		23.2 ± 0.92	22	< 0.001
Vehicle	3	29.8 ± 0.83	- -	
AD6		26.0 ± 0.82	13	< 0.01

Time course of the analgesic activity of AD6 by i.p. administration in the mouse at different time intervals before testing

Dosage: 0.1 mg/kg, i.p.

N. 6 animals/group

Table 12

KINETICS OF THE ANALGESIC ACTIVITY IN THE MOUSE
(WRITHING TEST INDUCED BY PHENYLQUINONE)

Treatment (i.v.)	Time (hrs)	Writhing (n° ± S.E.)	Inhibition %	p Student-t
Controls	0.08	29.3 ± 0.95	- -	
AD6		6.3 ± 0.42	78	< 0.001
Controls	0.16	29.2 ± 0.87	- -	
AD6		7.0 ± 0.63	76	< 0.001
Vehicle	0.5	30.0 ± 0.82	- -	
AD6		8.3 ± 0.76	72	< 0.001
Vehicle	1	29.5 ± 0.50	- -	
AD6		11.2 ± 0.79	62	< 0.001
Vehicle	2	28.7 ± 0.88	- -	
AD6		17.2 ± 0.79	40	< 0.001
Vehicle	4	29.5 ± 0.92	- -	
AD6		23.3 ± 0.88	21	< 0.01

Time course of the analgesic activity of AD6 by i.v. administration in the mouse at different time intervals before testing

Dosage: 0.01 mg/kg, i.v.

N. 6 animals/group

association with other pharmacologically acceptable drugs, using different pharmaceutical formulations as follows: Example 1) a capsule contains:

Active ingredient:	
- cloricromene hydrochloride	100.00 mg
Excipients:	
- sucrose	92.77 mg
- starch	30.93 mg
- magnesium stearate	34.60 mg
- povidone	25.48 mg
- monobasic potassium phosphate	20.80 mg
- trimethylate cellulose acetate	95.42 mg
- gelatine capsule	77.00 mg

Example 2) a vial of lyophilized compound contains

Active ingredient:	
- cloricromene hydrochloride	30.00 mg
Excipients:	
- mannitol	30.00 mg
One vial of solvent contains:	
- sodium chloride	45.00 mg
- water for injection, q.s. to	5 ml

Example 3) a suppository contains:

Active ingredient:	
- cloricromene hydrochloride	50.00 mg
Excipients:	
- semisynthetic glycerides, q.s. to	2 g

Example 4) (transdermal application) a patch contains

Active ingredient:	
- cloricromene base	200.00 mg
Excipients:	
- absorption enhancer	q.s.
- oleic base	q.s.

The transdermal patch consists of a reservoir containing the pharmaceutical preparation, a cutaneous adhesive and a non-permeable membrane.

Example 5) a cream contains:

Active ingredient:	
- cloricromene hydrochloride	5,00 g
Excipients:	
- primary emulsifier	2.50 g
- secondary emulsifier	0.80 g
- neutral oil	5.00 g
- glycerol	6.00 g
- water for injection q.s; to	100.00 g

Example 6) an unguent contains:

Active ingredient:	
- cloricromene base	5.00 g
Excipients:	
- lipid base for absorption	100.00 g
- neutral oil q.s. to	100.00 g

Example 7) a cutaneous gel contains:

Active ingredient:	
- cloricromene hydrochloride	5.0 g
Excipients:	
- solubilizing agent	20.00 g
- absorption enhancer	10.00 g
- gelifying agent	7.00 g
- neutral oil q.s. to	100.00 g

This invention being thus described, it is obvious that these methods can be modified in various ways. Such modifications are not to be considered as divergences from the very spirit and purpose of the invention, and any modification that would appear evident to an expert in the field comes within the scope of the following claims.

WE CLAIM:

1. A pharmaceutical composition which comprises as an active ingredient, an effective amount of 8-chloro3(beta-diethylaminoethyl)-4-methyl-7-ethoxycarbonylmethoxy coumarin or a pharmaceutically acceptable salt thereof, for the treatment of pathological conditions involving high release of nitric oxide (NO).

2. The composition according to claim 1 containing, as an active ingredient, 8-chloro-3-(beta-diethylaminoethyl)-4-methyl-7-et hoxycarbonyl-methoxy coumarin or a pharmaceutically acceptable salt thereof, for the treatment of a pathological condition which results in vasodilation and/or tissue damage.

3. The composition according to claims 1-2 containing, as an active ingredient, 8-chloro-3-(beta-diethylaminoethyl)-4-methyl-7-et hoxycarbonyl-methoxy coumarin or a pharmaceutically acceptable salt thereof, for the treatment of pathological conditions according to either of claims 1-2, which results in pulmonary inflammation, edema, erythema, dermatitis, psoriasis, skin ulcers, arthrosis, rheumatoid arthritis and other autoimmune diseases, hypotensive shock, septic shock, hypovolemic shock; vascular diseases, such as inflammations deriving from thrombophlebitis, hemorrhoids, or ulcerative colitis.

4. The pharmaceutical composition according to any of claims 1-3, wherein a pharmacologically effective dose of the active ingredient is associated with pharmacologically acceptable excipients and diluents.

5. The composition according to any of claims 1-4, for oral administration.

6. The composition according to any of claims 1-4, for parenteral administration.

7. The pharmaceutical composition according to any of claims 1-4, for topical administration.

8. A method for treating diseases associated with nitric oxide overproduction which comprises administering to a patient in need of said treatment, an effective amount of 8-chloro-3(beta-diethylaminoethyl)-4-methyl-7-ethoxycarbonyl methoxy coumarin.

9. The method according to claim 8, wherein said pathological conditions such as, for example, those associated with pulmonary inflammation, edema, erythema, dermatitis, psoriasis, skin ulcers, arthrosis, rheumatoid arthritis and other autoimmune diseases, hypotensive shock, septic shock, hypovolemic shock; vascular diseases, such as inflammations derived from thrombophlebitis, hemorrhoids, and ulcerative colitis, wherein a pharmacologically active dose of 8-chloro-3 (beta-diethylaminoethyl)-4-methyl-7-ethoxycarbonyl-methoxy coumarin or a salt thereof, is administered alone or in association with other acceptable drugs.

10. A therapeutic method for the treatment of pathological conditions involving high releases of nitric oxide which comprises administration of a pharmacologically effective amount of 8-chloro-3-(beta-diethylaminoethyl)-4-methyl-7-ethoxycarbonyl-methoxy coumarin or a salt thereof, to a patient in need thereof.

11. The therapeutic method according to claim 8, for oral administration.

12. The therapeutic method according to claim 8, for parenteral administration.

13. The therapeutic method according to claim 8, for intravenous administration.

14. The therapeutic method according to claim 8, for topical administration.

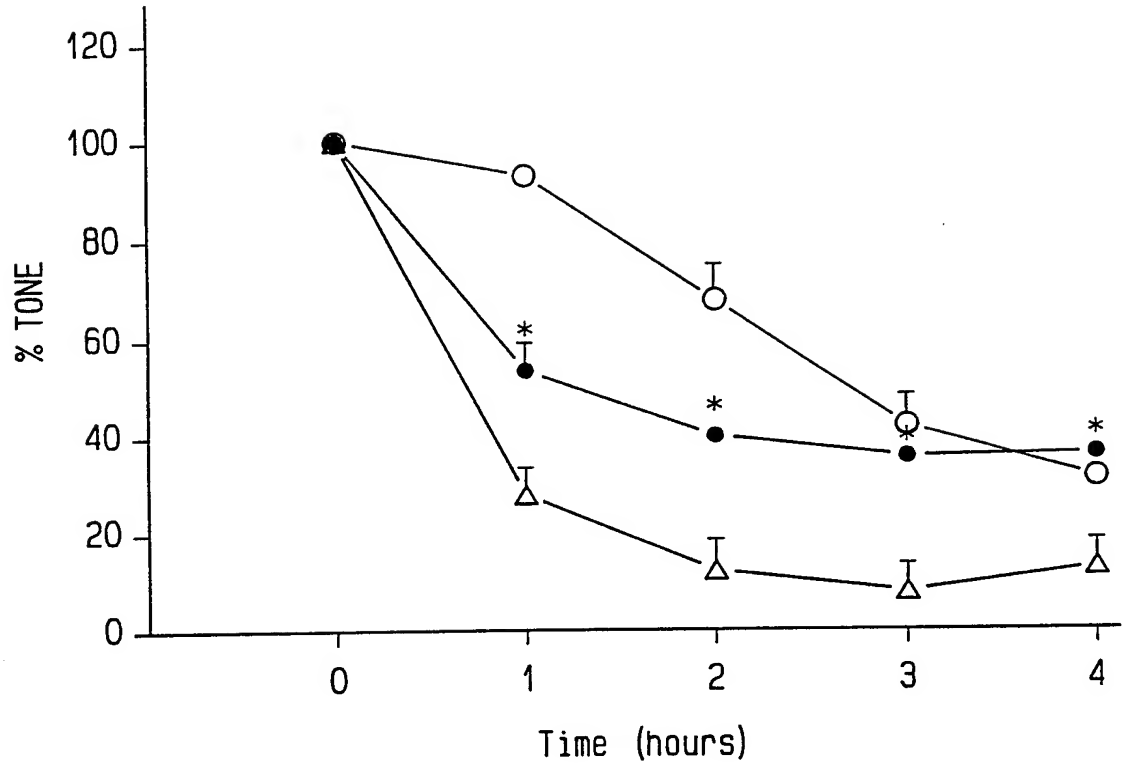

Fig. 1

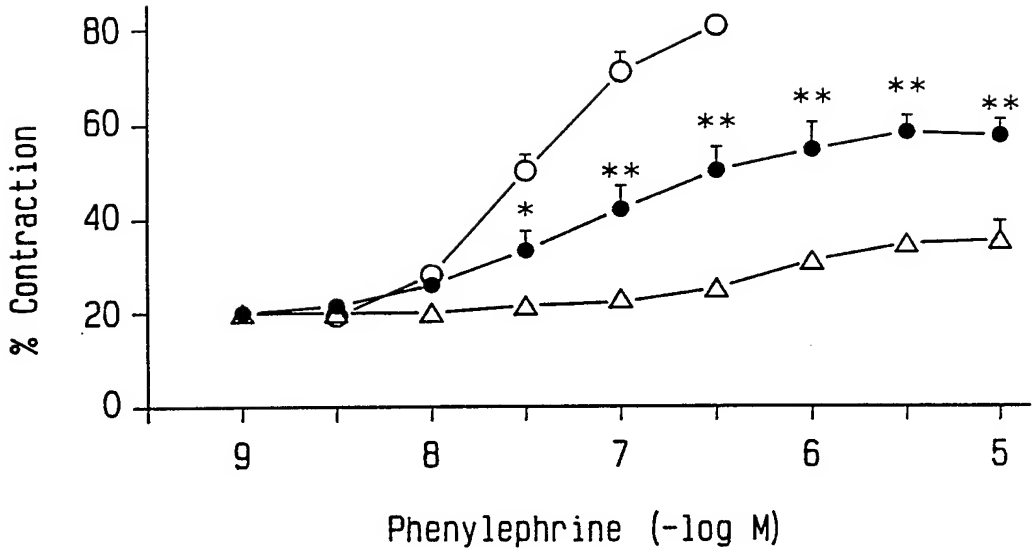

Fig.2

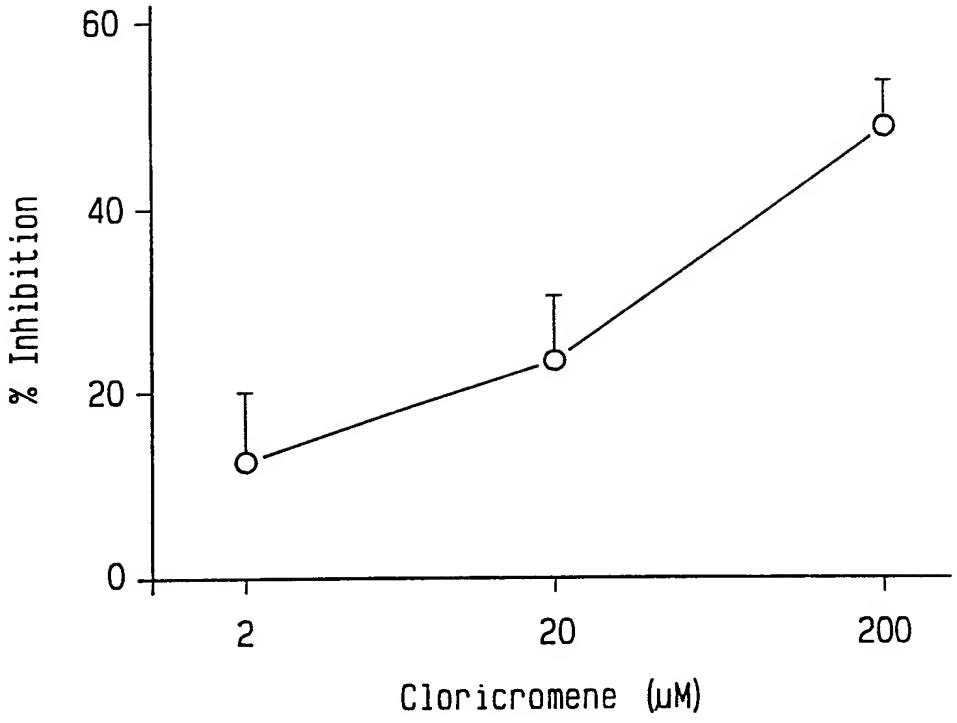

Fig.3

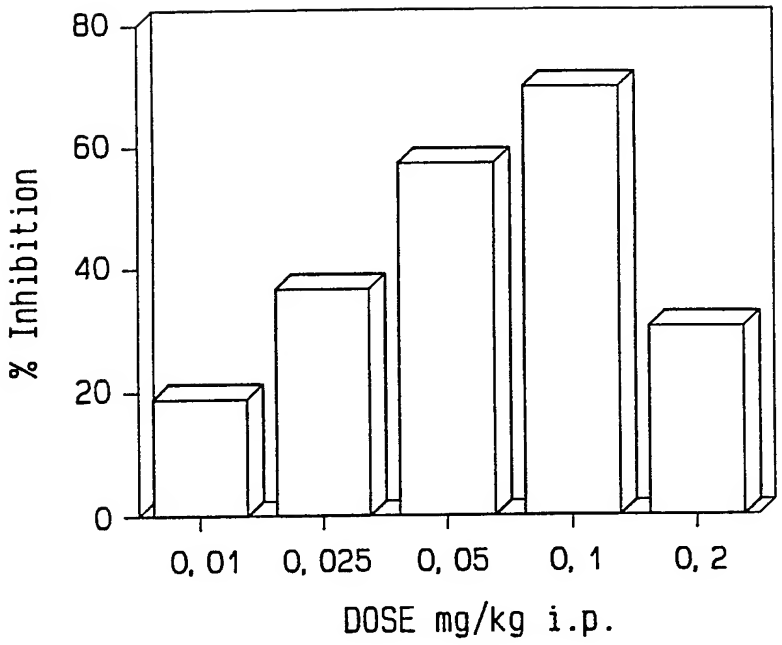
80
60
40
20
0
% Inhibition
0, 01
0, 025
0, 05
0, 1
0, 2
DOSE mg/kg i.p.

Fig. 4

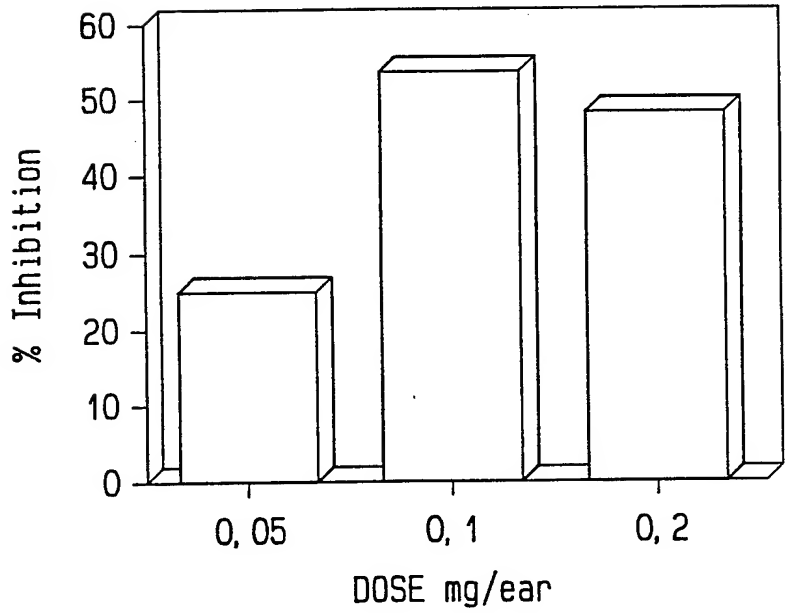
60
50
40
30
20
10
0
% Inhibition
0, 05
0, 1
0, 2
DOSE mg/ear

Fig. 5

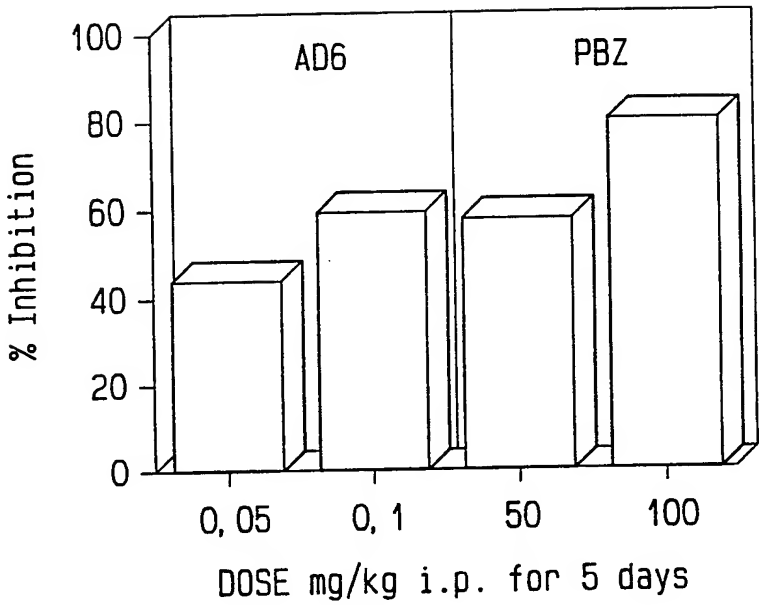
100
80
60
40
20
0
% Inhibition
AD6
PBZ
0, 05
0, 1
50
100
DOSE mg/kg i.p. for 5 days

Fig.5a

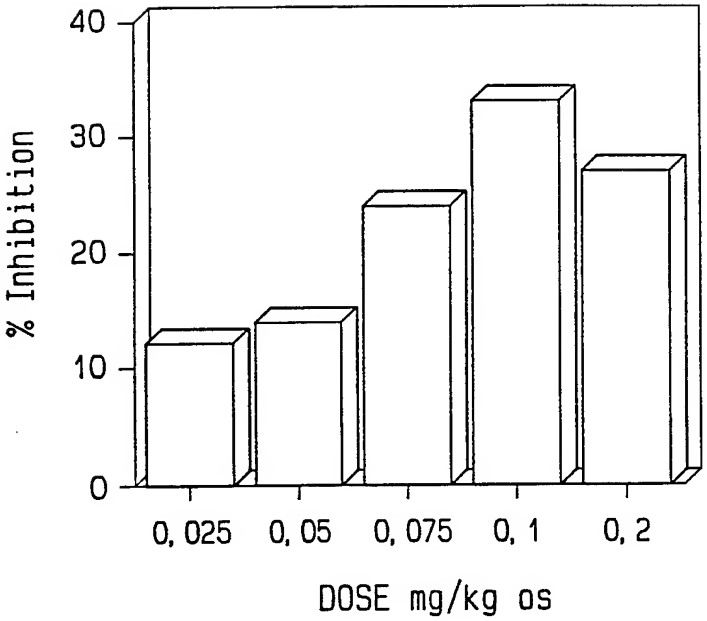
40
30
20
10
0
% Inhibition
0, 025
0, 05
0, 075
0, 1
0, 2
DOSE mg/kg os

Fig.6

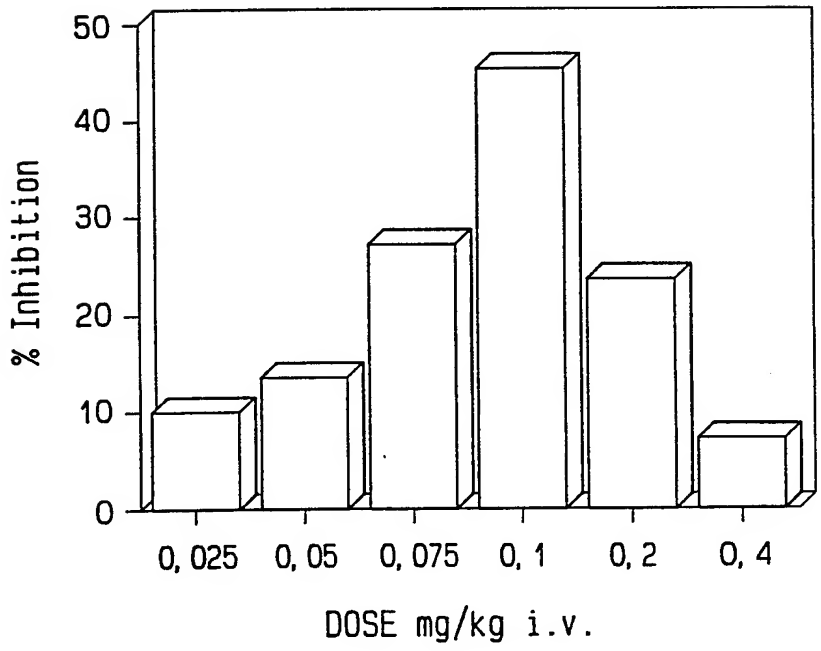
50
40
30
20
10
0
% Inhibition
0, 025
0, 05
0, 075
0, 1
0, 2
0, 4
DOSE mg/kg i.v.

Fig.7

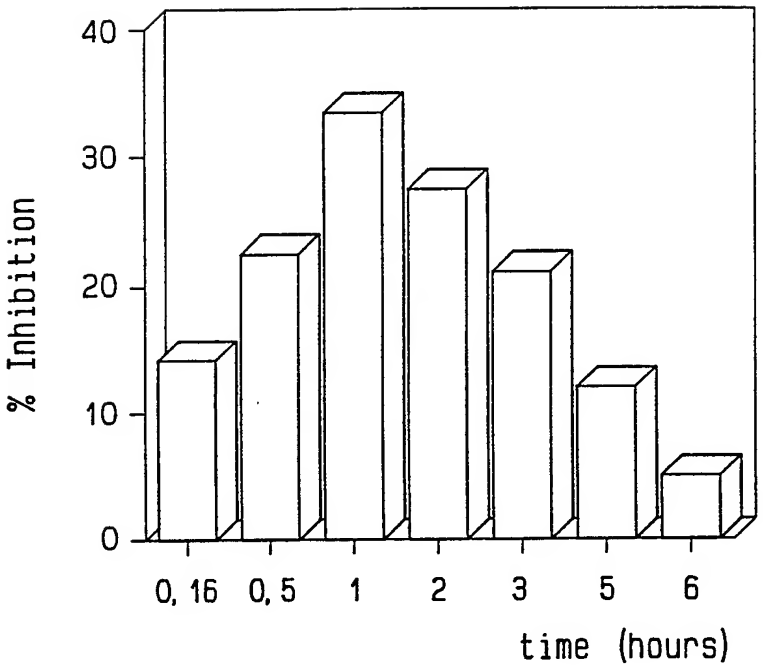

Fig.8

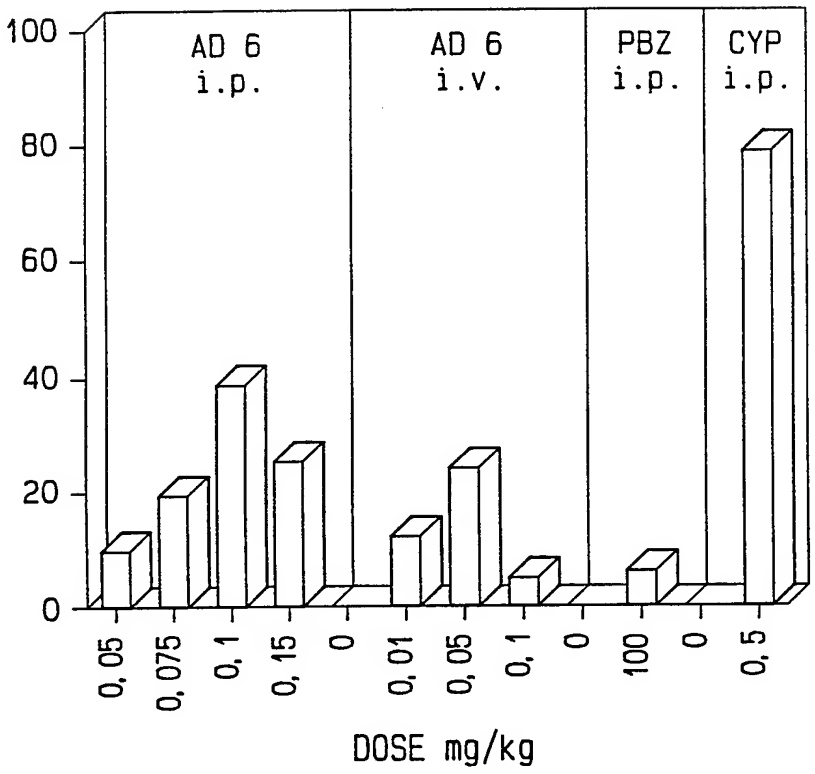
100
80
60
40
20
0
AD 6
i.p.
AD 6
i.v.
PBZ
i.p.
CYP
i.p.
0,05
0,075
0,1
0,15
0
0,01
0,05
0,1
0
100
0
0,5
DOSE mg/kg

Fig.8a

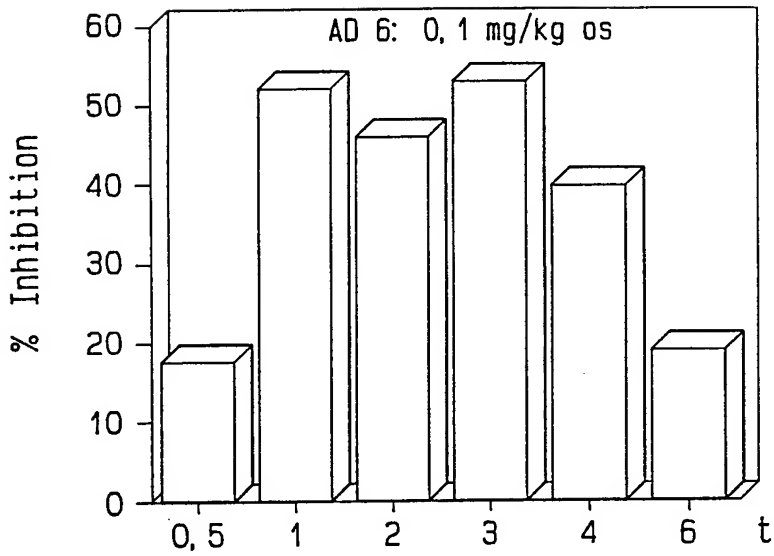

Fig. 9

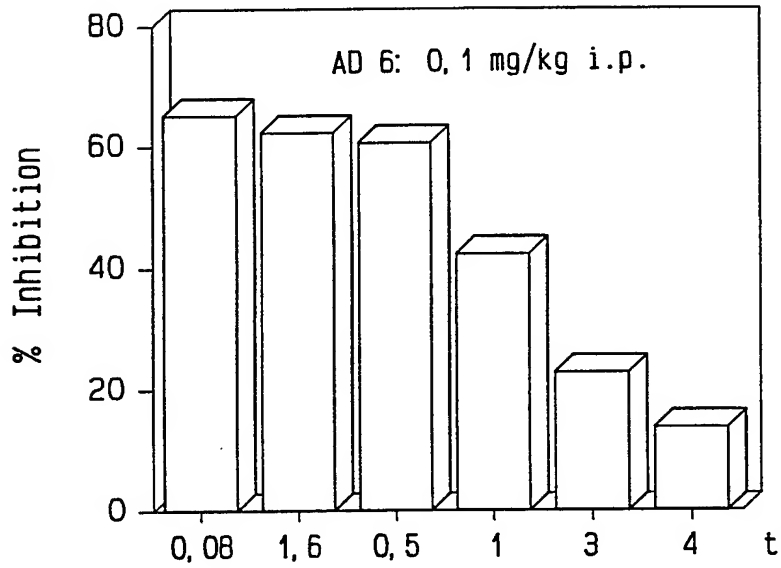

Fig. 10

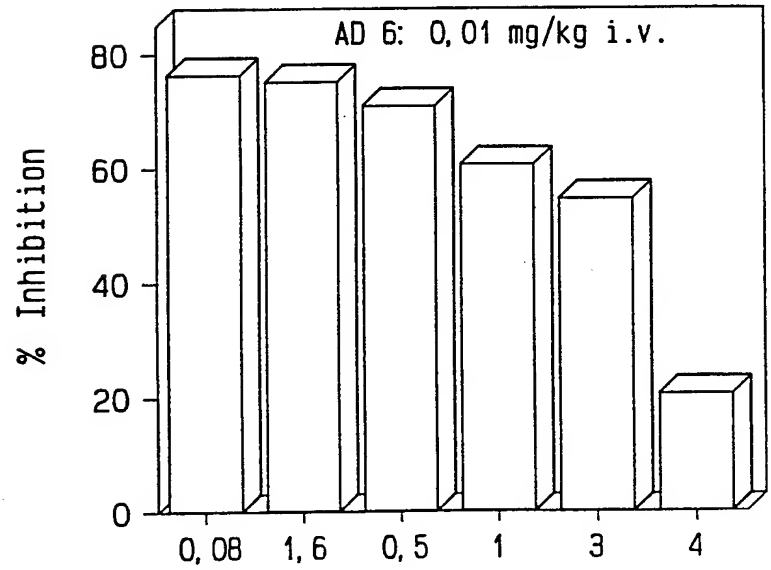

Fig. 11

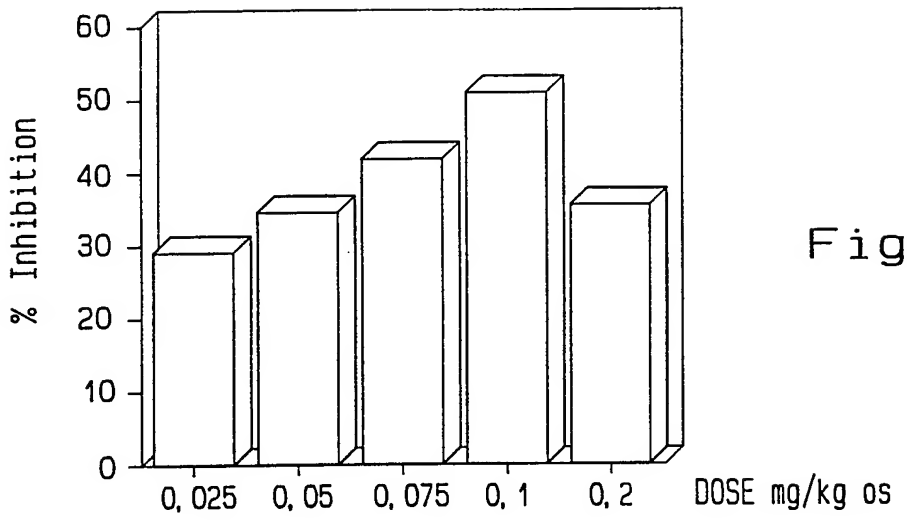

Fig. 12

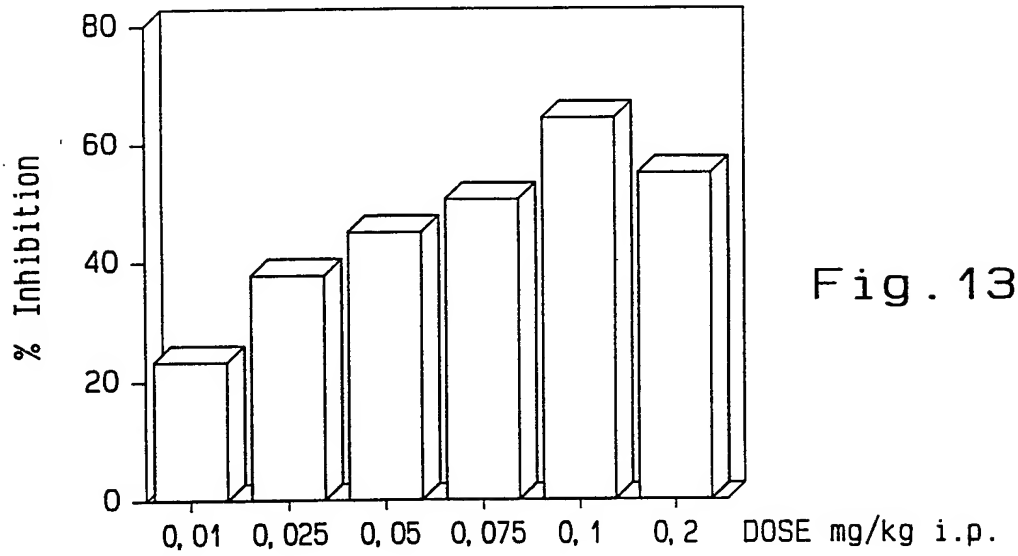

Fig. 13

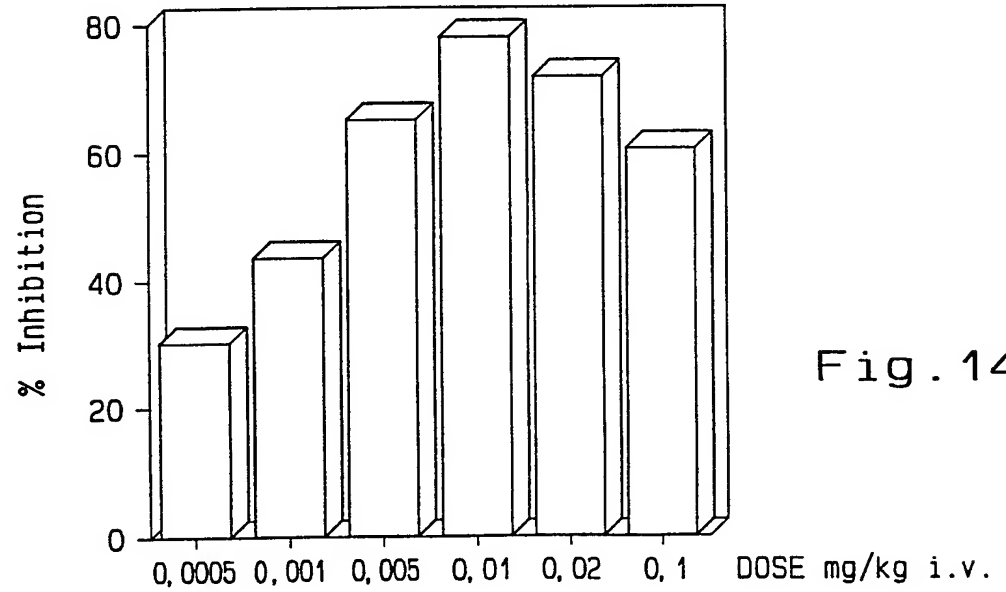

Fig. 14

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/EP 94/02008

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K31/37

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US,A,4 452 811 (DELLA VALLE) 5 June 1984 cited in the application see the whole document ---	1-14
P,X	EUROPEAN JOURNAL OF PHARMACOLOGY vol. 243, no. 2 , 19 October 1993 pages 107 - 111 B. ZINGARELLI ET AL. 'Cloricromene inhibits the induction of nitric oxide synthase' see the whole document --- -/--	1-14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
30 September 1994	21. 10. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Foerster, W

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. CARDIOVASC. PHARMACOL. vol. 17, no. 2 , 1992 pages 261 - 266 R. STURNIOLO ET AL. 'Protective effect of cloricromene, a coumarine derivative, in hypovolemic hemorrhagic shock in the rat' see abstract	1-10
X	EUROPEAN JOURNAL OF PHARMACOLOGY vol. 210, no. 2 , 1992 pages 107 - 113 F. SQUADRITO ET AL. 'Cloricromene, a coumarine derivative, protects against lethal endotoxin shock' see abstract	1-10
X	EUROPEAN JOURNAL OF PHARMACOLOGY vol. 198, no. 1 , 1991 pages 97 - 100 A. ZATTA ET AL. 'Polymorphonuclear leukocyte-dependent modulation of platelet function: Effect of cloricromene' see page 100	1-10
X	LIFE SCIENCES vol. 51, no. 26 , 1992 G. CALAPAI ET AL. 'Cloricromene antagonizes antidipsogenic effects induced by endotoxin, but not by TNFalpha, in the rat.' see the whole document	1-10
A	EUROPEAN JOURNAL OF PHARMACOLOGY vol. 222, no. 2-3 , 1992 pages 181 - 184 V. MOLLACE ET AL. 'Cloricromene synergizes with antiplatelet drugs and nitric oxide-like factor derived from rat peritoneal polymorphonuclear cells' see the whole document	1-14

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/EP 94/02008

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A-4452811	05-06-84	AT-B-	371815	10-08-83
		BE-A-	871315	15-02-79
		CA-A-	1100498	05-05-81
		CH-A-	635334	31-03-83
		DE-A,C	2846083	23-05-79
		FR-A,B	2412541	20-07-79
		FR-A,B	2412542	20-07-79
		GB-A,B	2008109	31-05-79
		JP-C-	1466145	10-11-88
		JP-A-	60243081	03-12-85
		JP-B-	63010154	04-03-88
		JP-A-	54079282	25-06-79
		NL-A-	7810946	21-05-79
		US-A-	4296039	20-10-81

PUB-NO: WO009500142A1

DOCUMENT-IDENTIFIER: WO 9500142 A1

TITLE: NEW PHARMACEUTICAL PREPARATIONS, CONTAINING 8-CHLORO-3 (betaDIETHYLAMINOETHYL)-4-METHYL-7-ETHOXYCARBONYLMETHOXY COUMARIN AND THE SALTS THEREOF, IN THE TREATMENT OF PATHOLOGICAL CONDITIONS INVOLVING HIG

PUBN-DATE: January 5, 1995

INVENTOR-INFORMATION:

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CIRILLO, ROCCO	IT
HORNBY, EDWARD JOSEPH	GB
PROSDOCIMI, MARCO	IT

ASSIGNEE-INFORMATION:

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PROSDOCIMI MARCO	IT

APPL-NO: EP09402008

APPL-DATE: June 20, 1994

PRIORITY-DATA: ITPD930141A (June 21, 1993)

INT-CL (IPC): A61K031/37

EUR-CL (EPC): A61K031/37

ABSTRACT:

A pharmaceutical composition which comprises as an active ingredient, an effective amount of 8-chloro-3(beta-diethylaminoethyl)-4-methyl-7-ethoxycarbonylmethoxy coumarin or a pharmaceutically acceptable salt thereof, for the treatment of pathological conditions involving high release of nitric oxide (NO).